Investigating changes in glutamate transporter expression in different cell types following injury

Research Thesis

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Abstract

Glutamate is the most common neurotransmitter in the brain, but excessive activation of glutamate receptors results in neuronal dysfunction and death, a process called excitotoxicity. Excitatory amino acid transporter (EAAT) proteins are responsible for reuptake of glutamate from the synapse, thus keeping extracellular glutamate levels low and preventing injury. EAAT1/GLAST and EAAT2/GLT-1 are prevalent in the brain and primarily expressed in glial cells. Previous work by the Kirby lab and others demonstrates that neural stem and progenitor cells (NSPCs) in the adult hippocampus also express EAAT1 and EAAT2, though the function of EAATs in NSPCs is poorly understood. Given the important role of EAATs in protection from excitotoxic injury, we studied EAAT expression patterns in mice given kainic acid (KA), which causes excessive glutamate release and seizures. Previous work in the Kirby lab shows that EAAT1 and EAAT2 mRNA decline in the dentate gyrus (DG) subregion of the hippocampus beginning 1 day after KA injury. However, it is unknown which cell populations are driving this tissue-level change. In the present study, we used EAAT2-GFP and EAAT1-dsRed transcriptional reporter mice to examine EAAT2 and EAAT1 transcription in the DG after KA injury. We quantified the mean GFP fluorescence intensity (FI) in the whole dentate gyrus, a region occupied by EAAT2-expressing astrocytes and NSPCs, and found no difference between KA-injured mice and controls 1,3 and 7 day post-injection. Similarly, GFP FI in the subgranular zone, a region predominantly occupied by NSPCs, did not differ from controls. We also quantified GFAP% area to assess astrogliosis and found a trend towards increased GFAP% area, which did not reach significance ($p = 0.149$), 1 day after injury. This is consistent with previous results in wild-type mice demonstrating a non-significant trend toward astrogliosis 1 day post-injury and significant astrogliosis beginning 3 days after injury. There was no significant difference in EAAT transcription in both EAAT1-dsRed and

EAAT2-GFP mice 3 and 7 days after injury, time points previously found to coincide with astrogliosis. Future analysis will examine these cell specific changes in fluorescent reporter expression using a combination of protein marker expression, cell morphology, and anatomical location to identify astrocytes and neural stem cells. We expect these studies to increase our understanding of whether reduced EAAT expression following KA injury occurs globally or in specific cell populations. Our findings could help refine treatment strategies for brain disorders involving glutamate dysregulation.

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Introduction

Glutamate is the main excitatory neurotransmitter in the brain, and prolonged activation of glutamate receptors results in neuronal damage known as excitotoxicity. Glutamate transporters are responsible for uptake of glutamate from the extracellular fluid in the brain and thus avoid unnecessary activation of glutamate receptors (Zhou et al. 2014). The glutamate clearance from the synapse maintains homeostasis by keeping the high temporal and spatial fidelity of the transmission through the use of glutamate transporters (Hanson et al. 2015).

The glutamate transporter gene family has five excitatory amino acid transporter (EAAT) members (EAAT1–EAAT5) and these transporters are expressed in the brain in highly specific regional and cell-type patterns (Kavanaugh 2008). Expression of both EAAT1 and EAAT2 is prominent in the dentate gyrus (DG) of the hippocampus, a brain region important for cognition (O'Donovan et al 2017). These two transporters, EAAT1 (GLAST) and EAAT2 (GLT-1), are substantially expressed by the astrocytic plasma membrane, providing these cells with an enormous capacity for glutamate uptake (Schousboe 2005).

Previous studies suggest that astrocyte-expressed EAAT2 is responsible for greater than 90% of glutamate transport and is the predominant glutamate transporter in the brain (Kim et al. 2011). Furthermore, An additional EAAT-expressing population unique to the adult DG are adult neural stem and progenitor cells (NSPCs). Preliminary data from my lab shows that NSPCs express both EAAT1 and EAAT2, but not other EAATs. But, it is not clear whether the EAAT expression by the cells (glial, astrocytes and NSPCs) occurs globally or it is cell specific. It would be interesting to look at how the expression changes from one cell population to other in the hippocampus after Kainic Acid (KA).

Kainic acid is often used as a model of excitotoxicity and causes uncontrolled release of glutamate(Wang et al. 2005). When injected, KA binds to kainate receptors and results in an initial latent period followed by the onset of the seizures (Hubbard et al. 2016). The seizure activity is monitored by observing the mice using a well recognized behavioral response following KA injection. This includes mice showing "staring" spells, head nodding, and facial movements in the first 30 mins. After one hour, the animal starts recurrent limbic motor seizures, forepaw tremors, rearing and loss of balance. (Zhang et al. 2011). Prolonged seizures to KA adversely impacts the hippocampus and results in neurotoxicity due to excess glutamatergic stimulation and thus, causes neuronal damage in hippocampus(Zhang et al. 2011).

My research focuses on EAAT expression by astrocytes and NSPCs following injury. I specifically analyzed the subgranular zone (SGZ) of the DG and the entire DG (combined hilus, SGZ, and granule cell layer) based on the location of these cells to detect how they change in the expression. I used transcriptional reporter mice for both EAAT2-GFP and EAAT1-dsRed to examine EAAT2 and EAAT1 transcription in the DG after KA injury (Regan et. al 2007). We looked at the expression in the whole dentate gyrus, a region occupied by EAAT2-expressing astrocytes and NSPCs. We also looked at the expression in the subgranular zone, a region predominantly occupied by NSPCs.

Previous work in the Kirby lab shows that EAAT1 and EAAT2 mRNA decline in the DG of the hippocampus beginning 1 day after KA injury. Preliminary data from my lab also shows that after 1D there is no reduction in the NSPCs expression between KA and Saline injected mice. Based on this data, We hypothesized that NSPCs won't change in their expression of EAAT1 or EAAT2 after KA instead we predict that astrocytes will reduce expression of EAAT1 and EAAT2.

Background

Neurological disorders are a critical health care issue and are the world's largest cause of disability-adjusted life years (DALYs), or years of healthy life lost to due to death or disability (Collins 2017). Studies shows that NSPCs are promising for reducing neurological disability, and thus have emerged as a potential therapeutic approach for neural repair (Casarosa et. al 2014).

Adult stem cells including NSPCs reside in specialized anatomical locations known as niches (Zhao et al. 2018). The NSPCs are self renewing, multipotent cells and have radial processes spanning the entire granule cell layer and ramifying in the inner molecular layer (Yao et al. 2012). The behavior and fate of stem cells are strongly influenced by their niche and in the adult brain, NSPCs are restricted only to certain brain regions (Zhao et al. 2018). Two major NSPC populations reside in the adult brain in the hippocampal DG and walls of the lateral ventricles. These NSPC populations undergo proliferation and generate new neurons which mature over several weeks in the DG and olfactory bulb respectively (Yao et al., 2012).

Neurogenesis can be affected by extrinsic stimuli like seizures, growth factors and neurotransmitters (Zhao et. al 2018) and intrinsic mechanisms including dysregulation in epigenetics and transcription factors (Johnson et. al 2009). These mechanism contributes to numerous diseases for instance pathological events like Traumatic Brain Injury(TBI) can alter the neuronal function and DG is vulnerable to these changes because the cells lose their ability to regulate neurogenesis (Ngwenya et. al 2018). The decrease in neurogenesis contributes to hippocampus dependent behavioral deficits, cognition and emotional decline often seen in neurological disorders like epilepsy and seizures (Braun et a. 2014).In my experiment, KA injections were used to cause seizures in mice. The change in the EAAT expression was looked at 1, 3 and 7 days post- injury. Two specific cell types, astrocytes and NSPCs, were looked at

following excitotoxicity as EAAT expression and glutamate transportation across synapse is important in the neurological disorders.

Preliminary Data

EAATs and their expression in the NSPCs

To look at the EAAT expression in the NSPCs, Kirby Lab mined a publicly available single cell RNA sequencing dataset (Shin et al., 2015; GEO: GSE71485) for expression of EAAT genes in NSPCs. Of the five EAAT transcripts, EAAT1(slc1a3) and EAAT2 (slc1a2) were enriched in NSPCs relative to the whole DG tissue (Figure 1) . EAAT1 expression was particularly robust in the NSPCs.

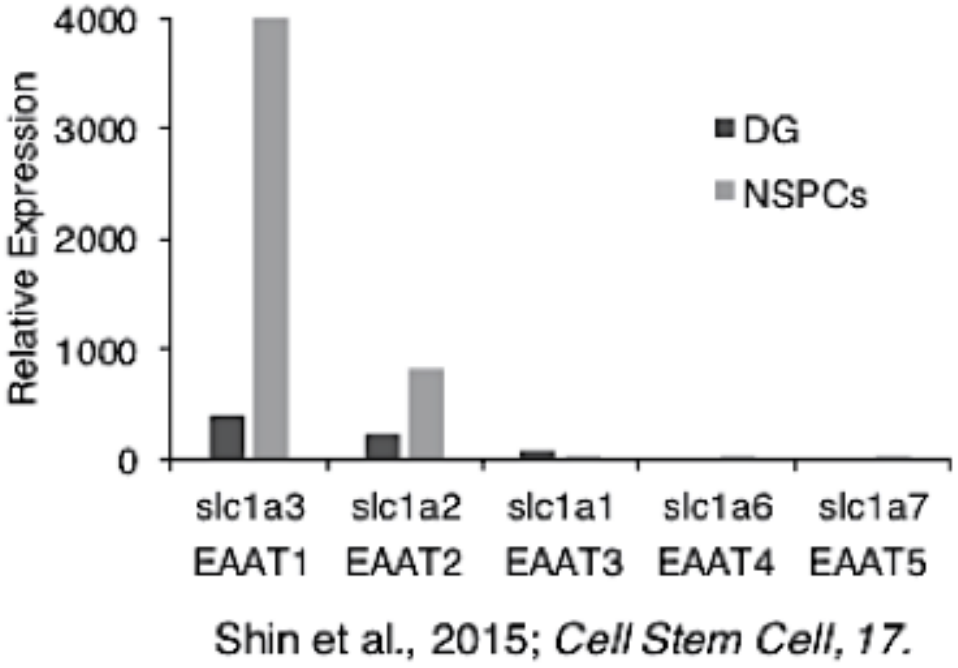


Figure 1. NSPCs express EAAT genes in vivo. Quantification of EAAT genes (slc1a3, slc1a2,slc1a1,slc1a6 and slc1a7) using RNA sequencing of Nestin-CFP expressing DG NSPCs (Shin et al. 2015)

EAAT mRNA expression declines following KA

Preliminary data from my lab also shows that following KA injury, EAAT expression declines in the whole DG tissue (Figure 2). It is unclear why EAAT1/ EAAT2 mRNA decline following injury. Since these transporters are expressed by NSPCs and astrocytes, it could be possible that one of these cell types, or both, is down regulating EAAT expression and my experiment aims to look at the expression of these cells following KA injury.

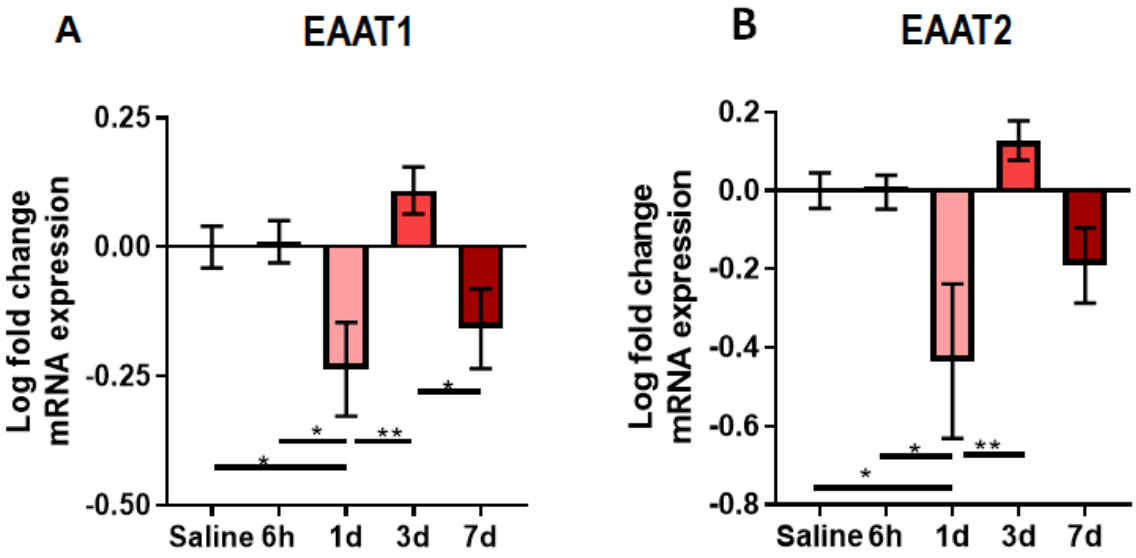


Figure 2.EAAT1 and EAAT2 mRNA decline in the DG following KA injury. Mice were injected with Saline or KA (25 mg/kg), and the DG were isolated for qPCR 6 hours or 1,3,7 days later. EAAT1 and EAAT2 mRNA significantly declined 1 day after KA. The EAAT1 and EAAT2 expression significantly increased at 3D and declined at 7D compared to Saline injected mice.

Methods

Animals:

EAAT1 and EAAT2 transcriptional reporter mice (Regan et al. 2007) were kindly gifted by Dr. Jeffrey Rothstein (Johns Hopkins University). Heterozygous EAAT1-Dsred mice and EAAT2-GFP mice were crossed with C57BL/6 wild type mice from The Jackson Laboratory. We used the offspring of 8-10 weeks old of these breeders for experiments. The offspring were genotyped using GFP primers and Dsred primers. The sequence of GFP and DsRed primers and their internal controls use are:

GFP **dsRed**

eGFP301-F GTGCAGTGCTTCAGCCGCTA dsRed904-F TCCAAGGTGTACGTGAAGCA

eGFP614-R TCGATGTTGTGGCGGATC dsRed1394-R TACTGCTCCACGATGGTGTAGT

Internal Control: Internal Control:

Rosa24500 CAGGACAACGCCCACACA IMR7338 CTAGGCCACAGAATTGAAAGATCT

Rosa21360 CTGGCTTCTGAGGACCG IMR7339 GTAGGTGGAAATTCTAGCATCATCC

Both male and female Dsred + and GFP + mice were selected for the experiment. The mice were separated into three groups using litter, age and sex. The experiment was done with a total of three cohorts to investigate the change in the EAAT expression after KA injury with each group represented below;

3D time point	3D KA	3D Saline	7D KA	7D Saline
Dsred	n=8	n=6	n=9	n=5
GFP	n=9	n=8	n=10	n=8

Mice were intraperitoneally injected with KA (25 mg/kg) or saline. The mice were observed for average of 3 hrs and seizure activity was recorded. All mice elicited seizure activity. The mice were placed in the colony room for the next 24 hrs and the perfusions were done at 1D, 3D and 7D post-injury. All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and approved by The Ohio State University Institutional Animal Care and Use Committee.

Histology

Mice were perfused with ice-cold phosphate buffered saline (PBS)and the brains were collected and stored in 4% paraformaldehyde (PFA) in 4°C to fix over night. The next day the brains were placed in 30% sucrose for at least 3 days . 40 um sections were obtained via microtome and were stored in cryoprotectant at -40°C until immunohistochemical (IHC) processing. A series of every twelfth section across the entire extent of the hippocampus was used for IHC.

Immunohistochemistry

The tissue collected through slicing was used in a 2-day IHC procedure. On the first day, sections were rinsed 3 * 5 mins in PBS. Sections were blocked using 0.1% normal donkey serum , 0.4 % Triton-X and PBS solution for 30 mins. Sections were then incubated overnight at 4°C with primary antibodies diluted in blocking solution. The source and dilutions for primary antibodies were as follows for EAAT2-GFP sections: goat anti-GFP (1:1000; invitrogen) , mouse anti-GFAP (1:1000; invitrogen), rat anti SOX2 (1:1000). The source and dilutions for primary antibodies were as follows for detecting EAAT1-dsRed: goat anti-Dsred(1:500; invitrogen), mouse anti-GFAP (1:1000; invitrogen), rat anti SOX2 (1:1000; invitrogen). The next day the sections were rinsed 3 * 5 minutes in PBS and incubated with fluorophore-conjugated secondary antibodies, all raised in donkey, as follows: anti gt 488 (1:500, invitrogen), anti ms 647 (1:500, invitrogen), anti

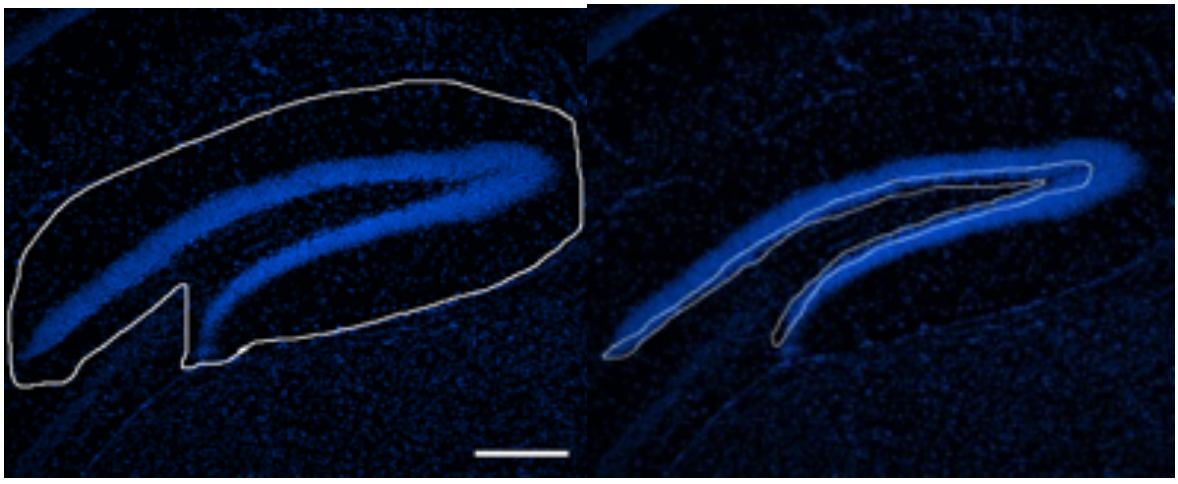
rt 594 (1:500, invitrogen)) in blocking solution. The sections were incubated in the nuclear stain Hoechst (1:2000) in PBS for 10 mins followed by 3 * 5 mins rinses in PBS. Section were then mounted on glass slides and cover slipped with Prolong Gold Antifade mountant (Invitrogen).

Microscopy

ll prepared slides were imaged using Zeiss apotome and Zen software. Z-stack images were obtained and the same exposure times were used across all subjects. . The sections were then analyzed using the software detailed below.

Fluorescence Intensity analysis

The fluorescence intensity of GFP and DsRed in the DG and SGZ of the hippocampus was determined using the NIH image J software to look at the level of fluorescence intensity. The Region of interest (ROI) was drawn around the molecular layer surrounding the whole DG. The SGZ ROI was drawn between the granule cell layer and hilus using the freehand tool.



The average pixel intensity was measured in the ROI to obtain mean fluorescence intensity and integrated density was measured to obtain the mean value of intensity with respect to area covered. The GFAP% area was calculated by applying a thresholding algorithm and measuring the thresholded GFAP+ area fraction. Different preset ImageJ algorithms were applied to EAAT1-

dsRed and EAAT2-GFP mice, but all images that were directly compared were thresholded using the same algorithm.

Statistical Analysis:

The data were then analyzed normalized to Control (Saline) and ANOVA test was done. To check the significance, Fisher's LSD post-hoc test was done with significance being $p<0.05$. The Statistics were run on 1, 3 and 7 Day post injury for both EAAT2-GFP and EAAT1-dsRed mice. The results were then shown using the graphs through GraphPad Prism software where one-way Anova test was done.

Pilot Data:

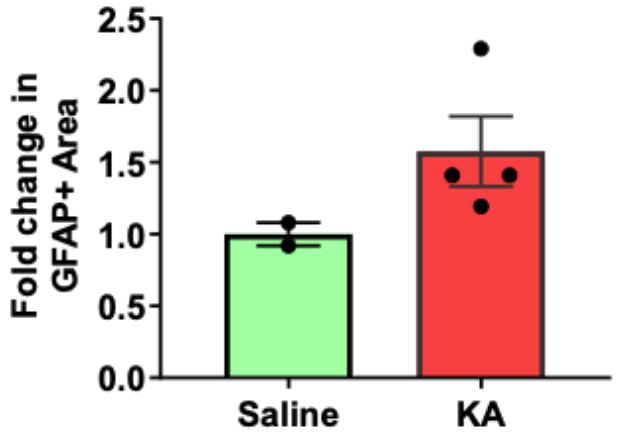


Figure 3. KA does not significantly increase astrogliosis 1 day post-injury. There is a trend towards increased GFAP %area in KA mice 1D post-injury, but this is not significant ($p>0.1$)

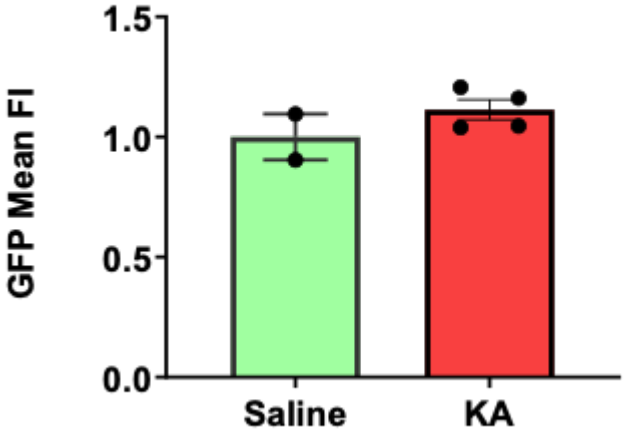


Figure 4. KA does not significantly increase EAAT2 expression 3 or 7 days post-injury. Mean GFP fluorescence intensity (FI) does not differ between KA and saline mice 1D post injury.

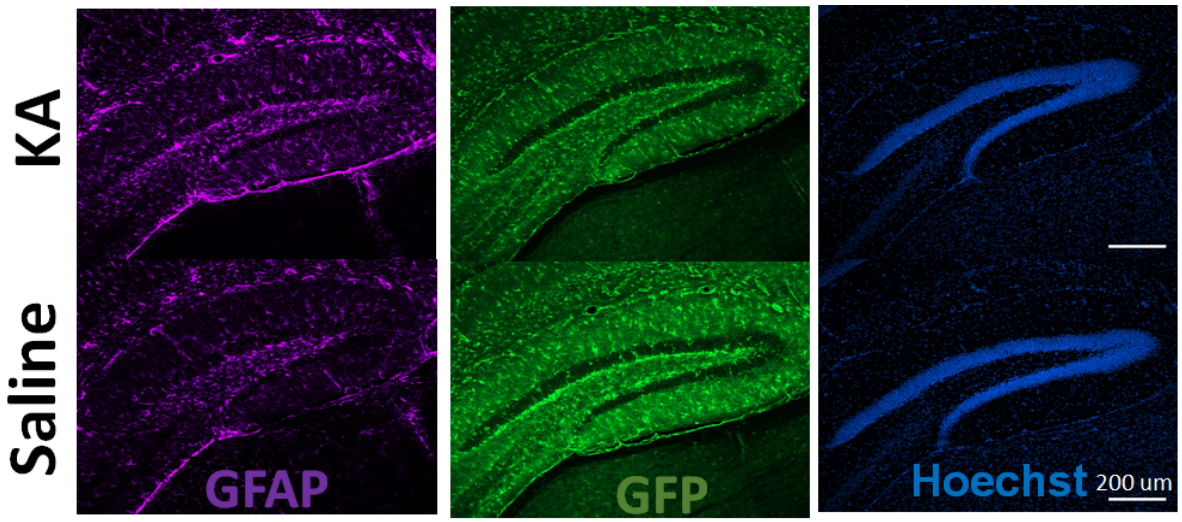


Figure 5. Representative images of KA and Saline injected mice 1D post-injury. GFAP, GFP, Hoechst staining to show the GFP and GFAP expression. Both the KA and Saline group shows similar expression post 1D injury

Results

My experiment focuses on studying the cell types that can contribute to the decline of EAAT expression following KA injury. To evaluate this expression, I used the transcriptional reporter mice. The pilot data indicated that KA does not significantly increase astrogliosis relative to Saline injected mice at 1D post injury (Figure 3). The pilot data also shows that the EAAT2 expression does not significantly change relative to Saline after KA injury at 1D (Figure 4). The representative image of both KA and Saline injected mice at 1D shows similar expression post ID injury (Figure 5). Based on this initial pilot data indicating no astrogliosis or change in EAAT2 expression response to KA, we decided to look at additional timepoints (3 and 7 days) for EAAT expression.

The EAAT1 driven expression was also evaluated using the dsred immunoreactivity in the EAAT1-dsRed mice. Both the KA and Saline injected mice shows similar expression at 3D and 7D relative to Saline (Figure 6). The EAAT1- Dsred expression was quantified to measure the average pixel intensity or Mean FI and the integrated density in the DG. The results showed no change in expression at 3 or 7 days after injury relative to Saline in the DG (Figure 7). We also quantified the Mean FI and Integrated density in the subgranular zone and did not find any significant change in expression at 3 or 7 days after injury relative to Saline in the SGZ (Figure 8). We also looked at the GFAP% area for EAAT1 and found that the GFAP % area in the EAAT1-dsRed KA mice trended towards increased astrogliosis at 3D (Figure 9), but did not reach significance.

The EAAT2 GFAP% area was also quantified and we found that KA does not significantly increase astrogliosis at 3 or 7 day post-injury (Figure 10). The representative images of GFP and GFAP shows similar expression between Saline and KA injected mice (Figure 11). The EAAT2

expression was quantified using the Mean FI and Integrated density and we did not found any significant change between the saline injected mice and 3 or 7 D KA injected mice in the DG (Figure 12). We also did not found any significant change in EAAT2 expression at 3 or 7 days after injury relative to Saline in the SGZ (Figure 13).

Overall, we did not detect a change in both EAAT1 and EAAT2 transcriptional reporter. The results indicated that KA and Saline group shows similar expression post 3 and 7D injury using the transcript model.

EAAT1- driven DsRed Expression

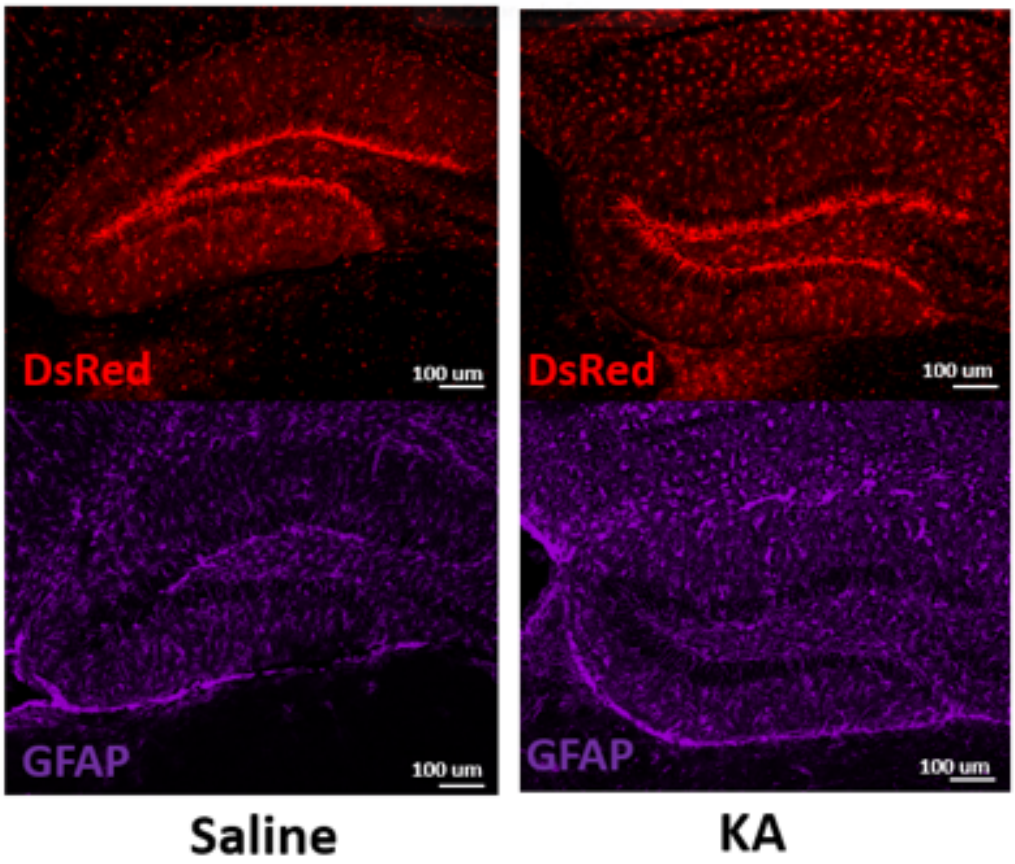


Figure 6. Representative images of KA and Saline injected mice 7-D post-injury. GFAP and Dsred staining to show the Glast and GFAP expression. Both the KA and Saline group shows similar expression post 3 and 7D injury

EAAT1- driven DsRed Expression

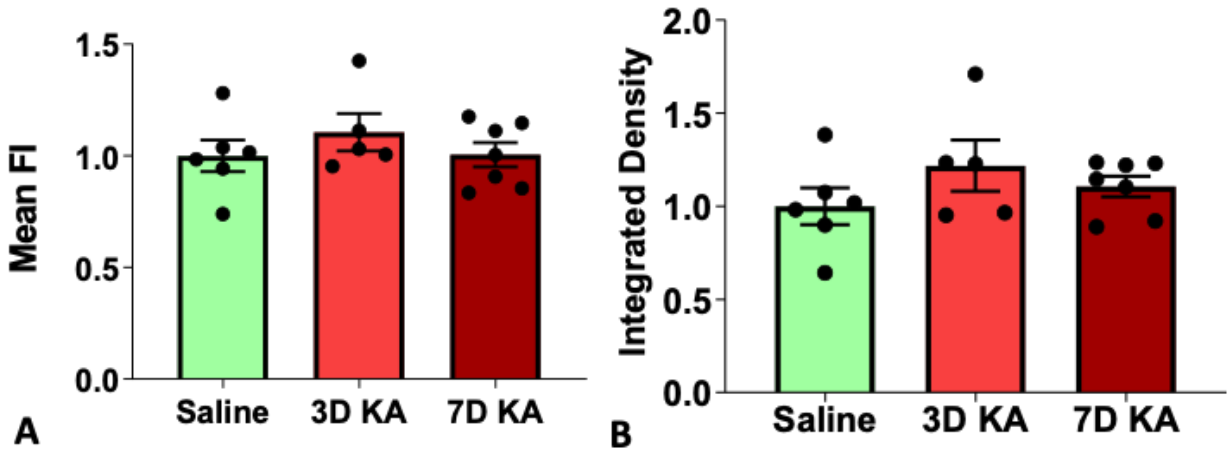


Figure 7. KA does not significantly increase EAAT1 expression 3 or 7 days post-injury in the DG.A) Mean DsRed fluorescence intensity (FI) between saline and KA mice. B) Integrated intensity (ID) between saline and KA mice in the DG

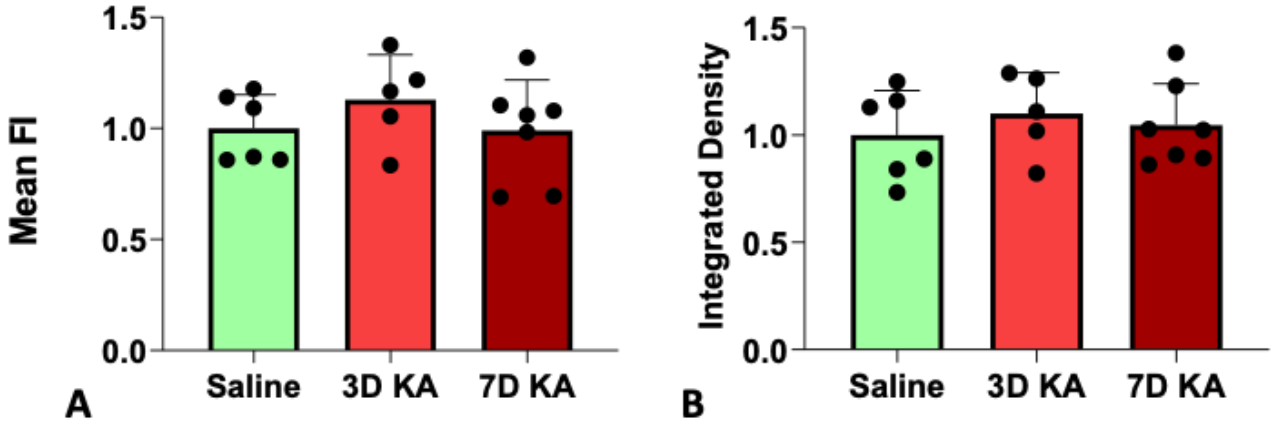


Figure 8. KA does not significantly increase EAAT1 expression 3 or 7 days post-injury in SGZ. **A).** Mean DsRed fluorescence intensity (FI) between saline and KA mice. **B)** Integrated intensity (ID) between saline and KA mice in the SGZ

KA does not significantly increase astrogliosis 3 and 7D post injury

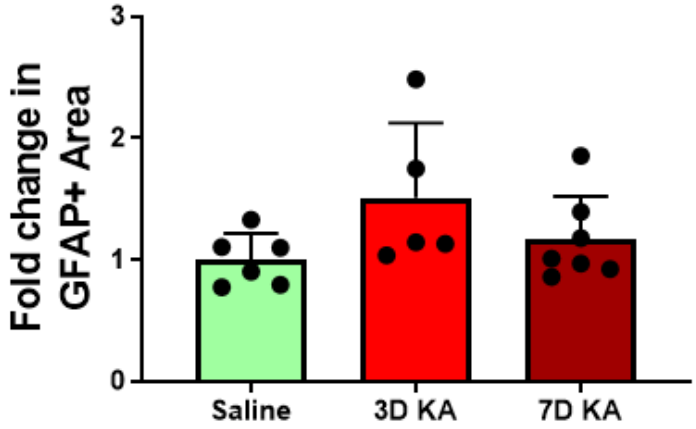


Figure 9.KA does not significantly increase astrogliosis 3 or 7 days post-injury. GFAP % area in the EAAT1-dsRed KA mice trended towards increased astrogliosis at 3D but did not reach significance.

EAAT2- driven GFP Expression

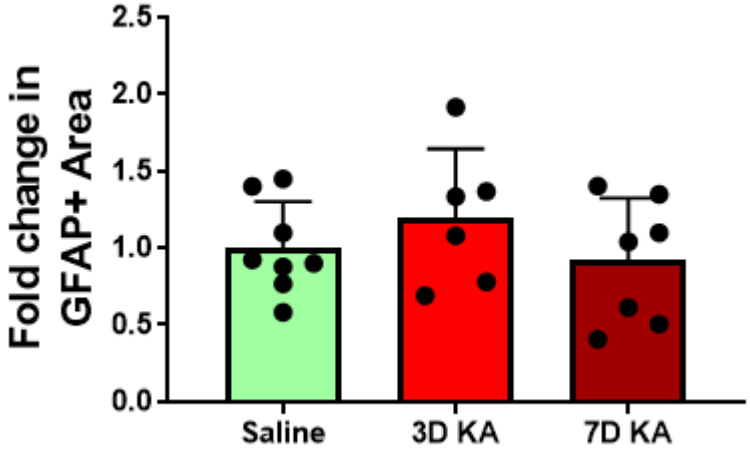


Figure 10.KA does not significantly increase astrogliosis 3 or 7 days post-injury. GFAP % area in the EAAT2-GFP KA does not differ significantly from the saline mice

EAAT2 driven GFP Expression

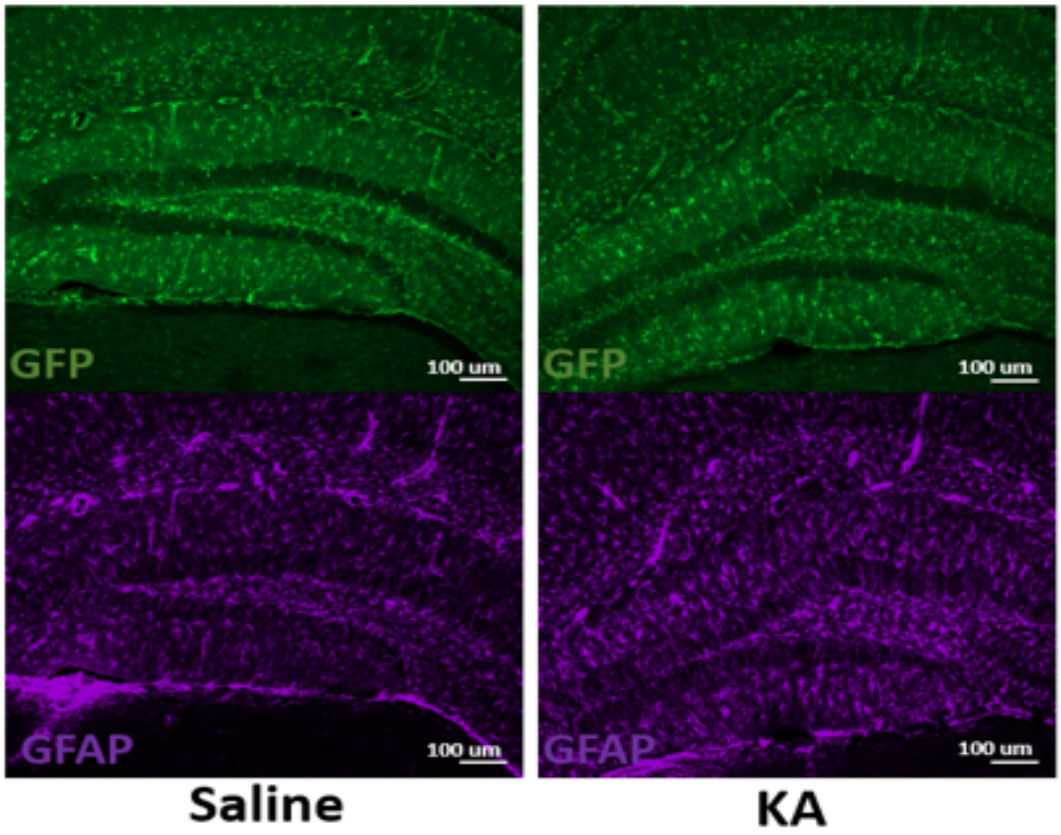


Figure 11. Representative images of KA and Saline injected mice 3D post-injury. GFP and GFAP staining to show the GFP and GFAP expression. Both the KA and Saline group shows similar expression post 3 and 7D injury

EAAT2 driven GFP Expression

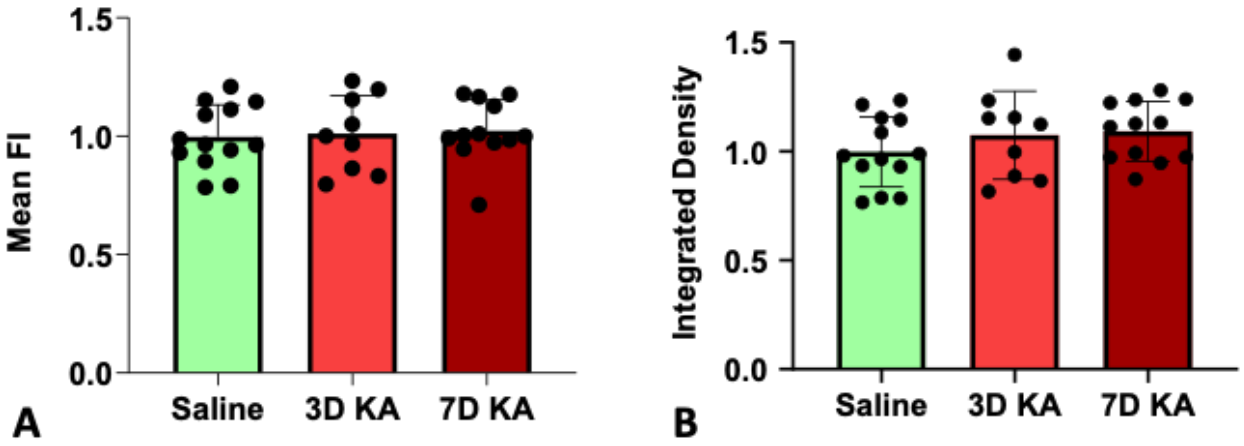


Figure 12. KA does not significantly increase EAAT2 expression 3 or 7 days post-injury in DG. **A)**Mean DsRed fluorescence intensity (FI) between saline and KA mice in the DG. **B)** Integrated intensity (ID) between saline and KA mice in the DG

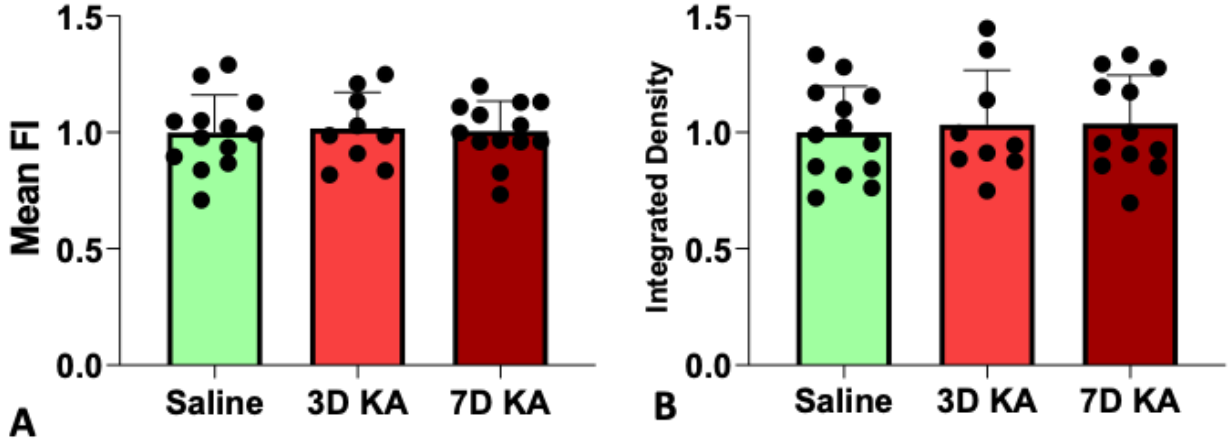


Figure 13. KA does not significantly increase EAAT2 expression 3 or 7 days post-injury in the SGZ. Mean DsRed fluorescence intensity (FI) between saline and KA mice in the SGZ. **B)** Integrated intensity (ID) between saline and KA mice in the SGZ

Discussion

The thesis investigated the changes in glutamate transporter expression in hippocampal subregions following injury. Based on the preliminary data from Kirby Lab suggesting that there is no reduction in EAAT expression in NSPCs at 1D post-injury, we hypothesized that NSPCs won't change in their expression of EAAT1 or EAAT2 after KA instead we predicted that astrocytes will reduce expression of EAAT1 and EAAT2. Results indicated that we did not detect changes in fluorescent reporter expression in the SGZ or whole DG driven by EAAT1 and EAAT2 transcription.

We found no significant change in EAAT reporter expression at 3 and 7 days after injury in the whole DG, suggesting that unlike with qPCR there was no tissue-level change in EAAT transcription using the transcriptional reporters. Similarly, we found no significant differences between saline and KA mice in the reporter expression associated with EAAT2 and EAAT1 transcription in the SGZ, a niche for NSPCs.

Furthermore, reactive astrogliosis was not observed in either EAAT1-DsRed or EAAT2-GFP mice, despite a non-significant trend toward astrogliosis in EAAT1-DsRed mice at 3D post injury. Lack of astrogliosis complicates the interpretation of the EAAT reporter data. In previous studies performed by my lab, we observed a robust injury response including upregulation of the cytokine TNFα and reactive astrogliosis 3D post injury. Lack of reactive gliosis in this study suggests that the injury may not have been robust enough to drive a change in EAAT expression. It is unclear from present study whether these cells types have significant role in the reduction of EAAT expression following the KA injury since the reactive gliosis and the transporter expression was not significant. There is currently no study in the literature that has examined the function of EAATs in NSPCs specifically or have looked at the EAAT expression in specific cell types like

NSPCs after injury. However, one study done on the human neocortex showed that following TBI, the EAAT2 expression decreased in the astrocytes (Frank et al. 2006). It is possible that the expression was similar in the DG, however our study did not detect it.

The qPCR data that showed the change in EAAT expression was done at a larger sample size. However, increasing the sample size in this case won't be effective as we did not see a robust change in injury response, suggesting that the issue could be with the methodology. One reason could be the protein stability. Dsred degrades at a much slower rate than the GFP. The half life for dsRed is 8.1 days and 3.8 days for GFP (Vladislav et al. 2003). This suggests that through the use of transcription reporter mice it is possible that we did not reach the protein degradation point and thus could not detect the change in expression.

For future studies, it will be interesting to look at additional time points. It will also be better to use a method that truly isolates the cell populations for example Fluorescence activated cell sorting (FACS), to study the cell specific expression patterns. Furthermore, the analysis could be done differently by drawing the ROIs around the individual's cells instead of the whole region.

Conclusions

We investigated the changes in EAAT expression in different cell types following KA injury because it is important to understand what was driving the change at the tissue level. However, we did not detect a change in EAAT transcriptional activity after Kainic acid injury for the transcriptional reporter mice. Overall, the transcriptional reporter mice expressed the GLAST/Dsred and GLT-1/GFP expression. 3D post injury showed a trend towards increase astrogliosis but this was not significant. The % GFAP detected the area covered by the astrocytes post 1D, 3D and 7D. The % area measured did not show an increase in astrogliosis at 1,3 or 7D after KA. Lack of astrogliosis indicates that the injury may not have been robust enough to cause a change in EAAT expression. I expected the aim to enhance our understanding of glutamate transporters and the role of NSPCs and astrocytes in the transporter expression following injury. Future studies can enhance our understanding of the role of these cell types in regulating the glutamate expression more and could potentially help understand the mechanism of change in expression to initialize the therapeutic interventions for neurological disorders like epilepsy and seizures.

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